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Genetic variation in the mitochondrial genome of the giant grouper *Epinephelus lanceolatus* (Bloch, 1790) and its application for the identification of broodstock

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ABSTRACT

Mitochondrial DNA (mtDNA) markers are ideal for the validation of maternal inheritance and the identification of brood-stock in aquaculture breeding programs. The complete mitochondrial genomes of 11 species of grouper are currently available at the GenBank. This study was directed towards the characterization of mtDNA loci which can be applied for identification of interspecific F1 hybrids developed from *Epinephelus fuscoguttatus* and *Epinephelus lanceolatus* in aquaculture breeding programs. DNA was extracted from the fin clip of one specimen of *E. lanceolatus* which the source of sperm for the artificial spawning of the interspecific F1 hybrid *E. fuscoguttatus* × *E. lanceolatus*. Specific primers were designed to amplify the DNA after comparative analysis of the mtDNA genomes available at the GenBank. The primers were applied to test for cross-amplification in F1 hybrids as well as in the maternal parent *E. fuscoguttatus* (Forsskål, 1775) and the genetically related species *Epinephelus coioides* and *Epinephelus corallicola* (Valenciennes, 1828). DNA sequence analysis revealed that the Malaysian variety of *E. lanceolatus* exhibited variation at 11 of the 13 ORFs when compared to the variety from Taiwan. A distinct segmented duplication was observed in the D-loop region which was determined to be unique to the *E. lanceolatus* specimen obtained from Sabah, Malaysia. Cross amplification of mtDNA loci in the groupers *E. fuscoguttatus*, *E. coioides*, *E. corallicola* and the F1 hybrid of *E. fuscoguttatus* × *E. lanceolatus* revealed distinct profiles for each of the species with a clear indication that mtDNA were inherited from the maternal parent of the F1 hybrid.. mtDNA loci can be applied by fish breeders to determine interspecific hybridization events.

1. Introduction

The giant grouper, *Epinephelus lanceolatus* is a high value fisheries resource which is increasingly being targeted by the commercial fishing industry. This has resulted in a decline in the range and size of wild populations (Heemstra and Randall, 1993; Zhu and Yue, 2008) and necessitated the development of captive breeding programs which serve as a source of seed for the aquaculture industry (Tucker et al., 1993). Traditional breeding practices rely on the selection of parental broodstock on the basis of phenotypes rather than genotypes which may eventually lead to inbreeding and the associated loss of fitness. Breeders have now turned to DNA molecular markers as an alternative to phenotypic selection (Liu and Cordes, 2004). Mitochondrial DNA (mtDNA) loci are ideal for the identification of haplotypes from wild populations for inclusion in fish breeding programs. Mitochondrial DNA comprises a double stranded circular molecule with a size that ranges from 16,418 to 16,795 in groupers. Vertebrate mtDNA is maternally inherited and exhibits a higher level of point mutations as compared to plant mtDNA (Dowling et al., 1996). The gene content comprises 37 genes which include 13 protein coding genes, two rRNA: 12S RNA and 16S rRNA, 22 tRNAs, and one control region (Zhu and Yue, 2008). The control region exhibits the highest level of nucleotide variation with the mtDNA genome and is composed of conserved sequence blocks (CSB), a hypervariable section (Lee et al., 2006; Craig et al., 2009; Nguyen et al., 2006) and a region comprising variable number tandem repeats (VNTR) (Chen et al., 2004) all of which have been applied for mitochondrial genotyping (Han et al., 2010). The unique features of mtDNA which include maternal inheritance, lack of recombination (Hurst et al., 1999; Oh et al., 2007; Liu and Cui, 2009), rapid mutation rates and compactness make it ideal for the identification of broodstock and the verification of parentage in F1 hybrids (Fujii, 2001). The ability of molecular markers developed for mtDNA from one species of grouper to cross-amplify in

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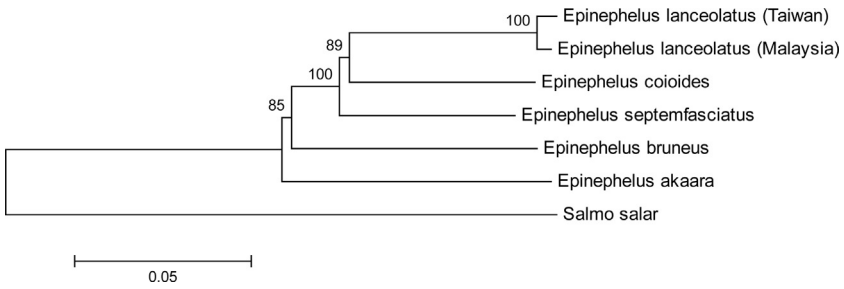


Fig. 1. Phylogenetic representation of the genetic relatedness between five species of *Epinephelus* using the neighbor joining (NJ) method. *Salmo salar* has been included as an outgroup. Numbers at the nodes represent bootstrap values (Replicates = 1000).

related species also facilitates the identification of species based on variation in amplification profiles. The complete mitochondrial genome of an *E. lanceolatus* specimen from Taiwan is currently available at the NCBI GenBank (FJ472837). Comparative analysis of the genomes of *E. lanceolatus* specimens from two geographically distinct regions can provide insights into mtDNA variation which can be exploited to develop molecular markers for the identification and selection of broodstock. The current study focused on genome resequencing of the mtDNA of an *E. lanceolatus* specimen from Sabah, Malaysia followed by comparative analysis with the existing reference sequence available at the GenBank.

2. Material and methods

2.1. Sample collection and DNA isolation

Broodstock of *E. lanceolatus, Epinephelus fuscoguttatus* and the F1 hybrids of *E. fuscoguttatus* (Female) × *E. lanceolatus* (Male) are currently being maintained at the Borneo Marine Research Institute, hatchery facility located at Universiti Malaysia Sabah. Ten live specimens of *Epinephelus coioides* and six of *Epinephelus corallicola* which comprise the broodstock were also selected for this study. DNA was extracted from fin clips of each of the parental genotypes and from 34 F1 hybrid specimens using the DNeasy extraction kit (Qiagen) and the concentration was adjusted to 50 ng/μl using sterile nuclease free water. DNA concentration and purity were verified using a Nanovue spectrophotometer (GE Life Sciences) (Broughton and Dowling, 1997; Rozen and Skaletsky, 2000).

2.2. Polymerase chain reaction (PCR)

Pairs of primers were designed for PCR amplification of the mtDNA genomes based on the sequences *E. lanceolatus* (FJ472837) accessed at the GenBank. A total of 13 primer pairs were designed to ensure complete coverage of the *E. lanceolatus* mtDNA genome (Table 1). PCR amplification was performed in final volume of 20 μl containing 1.2 μl $MgCl_2$ (1.5 mM), 0.4 μl dNTPs (0.2 mM each), 4 μl 1× Go*Taq* buffer (Promega), 1 U *Taq* DNA polymerase (Promega), 1 μl of each primer (5 μM), 2 μl template DNA and nuclease free water. Amplification was performed using a thermal cycler (MJ research, PTC-200) under the following conditions: pre-denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation (30 s at 95 °C), annealing (40 s at 56 °C), extension (2 min at 72 °C) and final extension (10 min at 72 °C). PCR products were resolved by electrophoresis on a 1.5% Tris-Boric Acid EDTA agarose gel, stained with Ethidium bromide and the gel was analyzed using a gel documentation system (Alpha Innotech, San Leandro, CA). The cross-amplification study in *E. coioides*, *E. corallicola*, *E. fuscoguttatus* and F1 hybrids of *E. fuscoguttatus* (Female) and *E. lanceolatus* (Male) was done under the same set of PCR conditions as stated above.

2.3. Cloning and sequencing

Individual PCR amplicons were cloned onto the pJET1.2/blunt cloning vector according to the manufacturer's instructions, transformed into chemically competent *Escherichia coli* (TOP10), followed by selection of positive colonies on Lysogeny agar containing Ampicillin (100 μg/ml). Single colonies were cultured in Lysogeny broth containing Ampicillin (100 μg/ml) for 16 h at 37 °C and plasmids were extracted using the GeneJET plasmid isolation kit (Fermentas). Plasmids were sequenced using the primers pJET1.2F and pJET1.2R (First Base Laboratories, Singapore).

2.4. Sequence assembly, annotation and analysis

Sequences were edited to remove vector contamination using the Vector screening software VecScreen available at the NCBI. Base calling was done using Phred. Assembly of sequences was done using the SeqManII software (DNASTAR Version 5.05). The genes order, gene content and gene location within the complete mitochondrial sequence and the comparative analysis were done using the sequence alignment algorithms blastx and blastn (Jenuth, 2000). Comparative analysis was done using the reference sequences of *E. lanceolatus* (Taiwan) (FJ472837), *Epinephelus akaara* (EU043377), *E. coioides* (EU043376), *Epinephelus septemfasciatus* (FJ594966) and *Epinephelus bruneus* (FJ594964). A neighbor-joining tree was constructed using MEGA 6.0 with *Salmo salar* (NC001960) as anvoutgroup. Secondary structure of the control region within mtDNA genome was determined using the Mfold server (Zuker, 2003).

3. Results

3.1. Genome organization

The complete mtDNA genome of the *E. lanceolatus* was amplified using 13 primer pairs (Table 1), the total length was determined to be 16,414 bp. The sequence was annotated and deposited at the GenBank and assigned accession number HQ660062. The order of genes was identical to that of the other *Epinephelus* species and the mtDNA genome was composed of 13 coding genes with 12 genes encoded on the H-strand and one gene (ND6) encoded on L-strand. The comparative analysis between gene sequences is listed in Table 2 and the order of genes is listed in Table 3.

3.2. Comparative analysis of mtDNA genome E. lanceolatus variety from Sabah and Taiwan

Comparative analysis of the mtDNA genome between *E. lanceolatus* variety from Taiwan and Sabah indicated a high degree of similarity at the nucleotide and amino acid level (Table 2) with variation observed at the ND5 gene. Phylogenetic reconstruction of the genetic relationships between five species of *Epinephelus* using the

Table 1
Locus name and primer sequences applied for the amplification of the entire *E. lanceolatus* genome and cross amplification in *E. fuscoguttatus*, F1 hybrids of *E. fuscoguttatus* (Female) and *E. lanceolatus* (Male), *E. coioides* and *E. corallicola*. (+) indicates positive amplification and (−) indicates no amplification.

Primers	Sequences 5′–3′	Cross amplification in *E. fuscoguttatus*	Cross amplification in *E. coioides*	Cross amplification in *E. corallicola*	Cross amplification in F1 hybrid *E. fuscoguttatus* X *E. lanceolatus*
ELMT1AF	ccctagaaatgcctcgaaaa	−	−	−	−
ELMT1AR	cctccgtgtaaaggaggtgt				
ELMT2AF	cacggaggaaatattcgttca	−	+	+	−
ELMT2AR	gcttaggccttttgcagtgt				
ELMT3AF	acatgttgatgtggcagagc	−	+	+	−
ELMT3AR	ggtttaacccccatgattcac				
ELMT4AF	cccctccagctccttagaaa	+	+	+	+
ELMT4AR	cactctcgcttagggctttg				
ELMT5AF	cacctcagggcttggtaaga	+	−	+	+
ELMT5AR	cttgacaaggcggggtaata				
ELMT7AF	agccaaccacataaccgttc	+	+	+	+
ELMT7AR	ggtggtcggtagtcaccaat				
ELMT8AF	gtgactaccgaccaccctca	−	−	+	−
ELMT8AR	atgtgctcaggttacggtca				
ELMT9AF	ccccttgaagttcctctcc	−	−	+	−
ELMT9AR	gcgagggttgtaggtcagag				
ELMT10AF	caacctccaaaacctcaacc	−	−	−	-
ELMT10AR	gggatttcaacccctgtttt				
ELMT11AF	tcttggtgcaactccaagtg	+	+	−	+
ELMT11AR	acggctaaggcggttagttt				
ELMT12AF	tgccttagaactcgcatcct	−	−	−	−
ELMT12AR	agcctgtctcgtgaaggaaa				
ELMTCIRF	aggagatccggacaacttca	+	+	−	+
ELMTCIRR	accaagcctttgtgttttcg				
ELMTCRF	cggtcttgtaaaccggatgt	−	−	−	−
ELMTCRR	taggaatctcaggggtttgg				

Table 2
Percentage of the nucleotide (Nt) and amino acid (AA) sequence identities of the *E. lanceolatus* genes comparison with their homologues of the other *Epinephelus* species.

Gene	*E.lanceolatus* (Taiwan)		*E. bruneus*		*E. akaara*		*E. coioides*		*E. septemfasciatus*	
	Nt(%)	AA(%)	Nt (%)	AA(%)	Nt (%)	AA (%)	Nt (%)	AA (%)	Nt (%)	AA(%)
12S rRNA	99	–	96	–	93	–	95	–	93	–
16S rRNA	99	–	94	–	92	–	94	–	92	–
NADH 1	99	99	89	98	84	96	88	98	85	96
NADH2	99	99	88	92	85	91	89	94	84	88
COI	99	99	90	99	86	98	89	99	86	98
COII	99		92	97	89	97	92	98	89	95
ATP8	99	98	87	85	84	82	83	78	79	73
ATP6	99	100	88	96	83	95	87	96	84	95
COIII	99	100	89	98	87	97	91	99	87	97
NADH3	99	99	87	96	84	93	86	95	87	97
NADH4L	99	100	89	96	90	93	90	94	88	95
NADH4	99	99	89	95	86	95	87	95	84	93
NADH5	94	96	87	93	83	90	89	94	85	92
NADH6	99	100	86	87	83	88	86	87	83	84
Cytb	99	99	86	94	82	93	87	93	83	92
Control region	97	–	73	–	73	–	78	–	75	–

Neighbor Joining (NJ) method (Fig. 1) revealed a well ordered tree, with strong bootstrap support, in which *E. coioides* was determined to be the most closely related to *E. lanceolatus*.

3.3. VNTR segment in control region

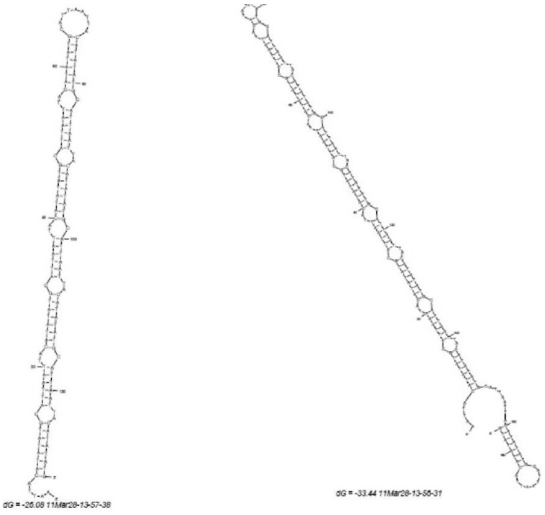
A distinct VNTR was observed in the control region with the Malaysian specimen containing 10 repeat units as compared to the one from Taiwan which contained 7. The sequence of the VNTR was similar in both cases and was represented by 5′ AATTACATATATG-CATT 3′. This repeat pattern appeared in the two individuals derived from Taiwan and Malaysia (Fig. 2).

3.4. Inheritance mode of mtDNA

PCR amplification profiles indicated that all 20 F1 hybrids developed from *E. fuscogutattus* × *E. lanceolatus* had inherited the mtDNA from the maternal parent. Five loci ELMT4, ELMT5, ELMT7, ELMT7 and ELMTCIR were specific to *E. fuscoguttatus* (Table 1) and to the F1 hybrids.

3.5. Cross-amplification in related species of groupers

Cross-amplification patterns in the closely related groupers indicated that 4 and 7 loci were specific to *E. coioides* and *E. corallicola* respectively (Table 1).



E. lanceolatus from Taiwan *E. lanceolatus* from Sabah

Fig. 2. The putative secondary structure of the VNTRs of *E. lanceolatus* from Sabah and Taiwan.

Table 3
Gene order of the mtDNA genome of the *E. lanceolatus* isolated from Malaysia.

Gene	Position
12S rRNA	70–1023
16S rRNA	1095–2802
NADH 1	2879–3853
NADH2	4069–5116
COI	5514–7064
COII	7221–7911
ATP8	7988–8155
ATP6	8146–8829
COIII	8829–9613
NADH3	9686–10,034
NADH4L	10105–10,401
NADH4	10395–11,775
NADH5	11994–13,832
NADH6	13829–14,350
Cytb	14428–15,571
Control region	15835–16,714

4. Discussion

4.1. Genome organization

The size of the mtDNA genomes of groupers ranges in size from 16,418 bp to 16,795 bp, the *E. lanceolatus* genome size was within this range (16,714 bp), although it was determined to be larger than that of the *E. lanceolatus* specimen from Taiwan by 140 bases as a result of the increase in the number of VNTR repeat units. A high similarity in each of the 13 coding genes when compared to other species of groupers provides a strong evidence for fixation. The low degree of non-synonymous variation within these protein coding regions is indicative of their positive selection within the genus.

4.2. Comparative analysis of mtDNA genome E. lanceolatus varieties from Sabah and Taiwan and related species of groupers

Comparative analysis of the mtDNA of the *E. lanceolatus* varieties from Sabah, Malaysia and Taiwan indicated a high level of the homology at the nucleotide and amino acid level with the exception of variation at the ND5 gene and the number of VNTRs thus providing evidence for a distinct haplotype. The ND5 gene is encoded on the light strand of the mtDNA. Variation at the ND5 gene has been reported earlier in Gadine species (Marshall et al., 2008; Brown et al., 2006) and provides evidence for selection at this particular locus. Although no evidence for positive selection was reported in Gadine species there was evidence of a structural variant. Mutations in the ND5 gene have been linked to exercise intolerance in humans as the structural protein is involved in the proton pump associated with mammalian mitochondria (Fonseca and Harris, 2008; Downham et al., 2008; Marshall et al., 2008), however no similar evidence has been found in fish. Analysis of this mutation will require sampling of a larger population in order to ascertain the degree of fixation and find evidence of positive selection and the associated increase in fitness. Phylogenetic analysis of the relatedness between species revealed that *E. coioides* is more closely related to *E. lanceolatus* as compared to *E. akaara*. Information pertaining to genetic relatedness is relevant to Grouper breeders who are intent on developing inter-specific hybrids and mitochondrial DNA can provide a preliminary basis for selecting species which are compatible for cross breeding. Species which have similar mtDNA genotypes are less likely to experience incompatibility which can arise as a result of genetic heteroplasmy.

4.3. VNTR segment in control region

The VNTR in the *E. lanceolatus* mtDNA was characterized by a distinct VNTR which was conserved in geographically isolated specimens, however the VNTR of the Malaysian specimen contained 10 repeat elements, whereas the specimen from Taiwan contained 7. Structural analysis of the VNTR using M-fold provided evidence of a distinct stem-loop structure (Fig. 1) which corroborates the stepwise mutation model, wherein, the propagation and expansion of repeat elements within a circular mtDNA genome is driven by DNA secondary structure. Such anomalies have been reported in Sturgeon (Brown et al., 1992) and VNTRs have also been reported to occur at the 5′ as well as the 3′ ends of mtDNA (Chen et al., 2004). Given the lack of recombination within the mtDNA genome (Hoarau et al., 2002; Faber and Stepien, 1998), and the lack of evidence of mtDNA heteroplasmy in groupers, the only likely explanation is DNA mismatch due to secondary structure formation during mtDNA replication. VNTRs can be applied for the DNA barcoding of *E. lanceolatus* brooders, since the number of repeat elements are distinct for each individual and the expansion can easily be estimated by PCR amplification using primer designed to flank the repeat regions.

4.4. Inheritance mode of mtDNA

The current study resolved the issue regarding mtDNA inheritance in F1 grouper hybrids. A clear pattern of maternal inheritance was observed in F1 hybrids based on the PCR amplification profile. The markers developed as a result of this study have application in the grouper hatcheries as mtDNA markers can now provide direct evidence of hybrid formation on the basis of a simple PCR test. Previous studies have focused on application of nuclear microsatellites (Rodriguez et al., 2006; Fernandez et al., 2000; Kim et al., 2004), however, cross-amplification of microsatellites in related species and binning of alleles provides a lower degree of accuracy in the case of parentage analysis from closely related hybrids.

4.5. Cross-amplification in related species of grouper

Primers developed for *E. lanceolatus* could amplify specific DNA loci in the closely related species *E. coioides* and *E. corallicola* however the amplification profiles were distinct in the case of each species. In the case of *E. coioides* only four of the 13 primer pairs amplified the mtDNA, these were located towards the 5′ terminal of the mtDNA and provides a strong evidence for conservation within this segment of the mtDNA, on the other hand, 7 of the 13 primers amplified loci in the mtDNA of *E. corallicola* indicating that

the species is more closely related to *E. lanceolatus* as compared to *E. coioides*. These findings can be applied to verify hybridization in F1 hybrids which are developed by crossing of different combinations of these two species.

5. Conclusion

Comparison of the mtDNA of two *E. lanceolatus* specimens from geographically distinct location revealed distinct variations in the ND5 gene and the control region. The number of VNTR repeats was found to have increased from 7 to 10 with the retention of the repeat element which is indicative of a stepwise mutation model. The mode of inheritance in F1 hybrids was determined to be maternal as evinced from the PCR amplification profiles. Cross-amplification patterns in closely related groupers implied that each species exhibited a distinct mtDNA fingerprint which could be applied to differentiate and identify the individual species.

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